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Original Article

ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and related Disorders Part 4 – Assay Validation and Quality Assurance

Teri Oldaker,[1]* Liam Whitby,[2] Maryam Saber,[3] Jeannine Holden,[4] Paul K Wallace,[5] and Virginia Litwin[6]

[1]Department of Flow Cytometry, Oldaker Teri—Genoptix Medical Laboratory, Carlsbad, California

[2]Department of Haematology, Royal Hallamshire Hospital, Liam Whitby—UK NEQAS for Leucocyte Immunophenotyping, Sheffield S10 2JF, UK

[3]Department of Flow Cytometry, Maryam Saber—Genoptix Medical Laboratory, Carlsbad, California

[4]Jeannine Holden, Beckman Coulter, Miami, Florida

[5]Department of Flow and Image Cytometry, Wallace Paul K—Roswell Park Cancer Institute, Buffalo, New York

[6]Virginia Litwin—Caprion Biosciences Inc, Montreal, Canada

Over the past six years, a diverse group of stakeholders have put forth recommendations regarding the analytical validation of flow cytometric methods and described in detail the differences between cell-based and traditional soluble analyte assay validations. This manuscript is based on these general recommendations as well as the published experience of experts in the area of PNH testing. The goal is to provide practical assay-specific guidelines for the validation of high-sensitivity flow cytometric PNH assays. Examples of the reports and validation data described herein are provided in Supporting Information. © 2017 International Clinical Cytometry Society

Key terms: standardization; flow cytometry; monocytes

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1. INTRODUCTION

Because of the ability to evaluate multiple parameters on individual cells with relatively high throughput, flow cytometry is an important platform for cellular analysis in both clinical and research laboratories. Information is generated regarding cellular lineage, development stage, activation state, and pathology from flow cytometric analysis. In the clinical laboratory setting, flow cytometry is routinely used for the diagnosis and monitoring of hematologic malignancies including minimal residual disease (MRD) determination, CD34 stem cell enumeration, lymphocyte immunophenotyping including CD4 T cell enumeration, paroxysmal nocturnal hemoglobinuria (PNH), as well as a wide variety of other applications (1).

PNH is a rare hematopoietic stem cell disorder resulting from the somatic mutation of the X-linked phosphatidylinositol glycan complementation Class A (PIG-A) gene (2). In PNH, there is a partial, or complete,

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Correspondence to: Oldaker Teri, Genoptix Medical Laboratory, Department of Flow Cytometry, Carlsbad, Los Angeles, CA Email: toldaker@cox.net

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Table 1
RBC PNH Panel

Table 1a. Panel Configuration

Fluorochrome	FITC	PE
Experimental tube	CD235a (clone 11E4B-7-6 (KC16)	CD59 (Clone MEM-43)

Table 1b. Reportable Results

b. Quasi-quantitative reportable results

Population	Immunophenotype	Reportable result
RBC Type I	$CD235a^{+}$, $CD59^{high}$	Relative percentage (%) of RBC
RBC Type II	CD235a, $CD59^{dim}$	Relative percentage (%) of RBC
RBC Type III	CD235a, $CD59^{-}$	Relative percentage (%) of RBC

Qualitative reportable results

Population	Immunophenotype	Negative for GPI marker (CD59)s	PNH interpretation (PNH clone)
RBC	$CD235a^{+}$	Yes/No	Present/Absent

Note that there is currently no single consensus panel for PNH Testing. The example shown is one of the recommended options[2], Validation data for this panel configuration is provided in Tables 3–9.

absence of expression of GPI-anchored proteins such as CD59 on red blood cells (RBC) and CD14, CD24, and CD157 on white blood cells (WBCs) (3,4).

ICCS Consensus (5) and Practical Guidelines (6) for detecting PNH phenotypes on RBC and WBC lineages have been published. RBC methodology consists of identifying RBC by the expression Glycophorin-A (CD235a) and then evaluating the expression of the GPI-linked protein, CD59 on CD235a-stained cells (Table 1, Fig. 1). As detailed previously (6,7) and in Section of this document, for WBC methods, the use of FLAER, an Alexa488-conjugated derivative of Pro-aerolysin, that binds to the GPI-moiety of most GPI-linked molecules is recommended.

Note that we use the more appropriate term "neutrophils" instead of the historical term "granulocytes" throughout this document. Modern gating techniques based on light scatter, CD45 and CD15 (see parts 2 and 3) result in exclusion of basophils and most eosinophils (although not bands) from the population of interest so that for the most part "granulocyte" clones are in fact "neutrophil" clones.

As the PNH testing community is transitioning to this more accurate terminology, we will use the term neutrophils for this document. As outlined in Section of this document, both neutrophils and monocyte lineages should be assessed for the presence of PNH phenotypes. Neutrophils are best delineated using CD15 and the expression of FLAER and the GPI-linked proteins, CD24 and/or CD157 are assessed (8). Monocytes are most efficiently delineated with CD64 and 'gated' monocytes assessed for binding of FLAER and the expression of GPI-linked proteins CD14 and/or CD157 (9) (Table 2, Fig. 2). In both the RBC and WBC assays, the PNH clone size is reported as the percentage of RBC, monocytes, or neutrophils expressing the PNH phenotype (i.e., a lack of expression of GPI-linked proteins) (9,10).

For the diagnosis and monitoring of PNH, flow cytometric methods are now considered the "gold standard" (5,7). The validation of PNH methods must follow the current recommendations for the validation of flow cytometric methods (8,11–13). Like any method validation, the validation process for PNH methods can be reduced to three general stages: (1) say it (the validation plan); (2) do it (the experimental phase); prove it (the validation report) (Fig. 3). In addition, it is essential that the method is validated under the same laboratory conditions that will be used during routine clinical testing. Thus, the assay must be fully optimized prior to initiating the validation process. The panel configuration, reagents, sample type, sample preparation, acquisition settings, and gating strategy should be documented in a method Standard Operating Procedure (SOP). In addition, the instrument qualification process should be documented and an instrument operating SOP in place (14,15).

In this manuscript, guidelines for PNH assay validation will be presented, and example validation data from a PNH RBC and WBC assay will be presented (Tables 1 and 2). The PNH RBC and WBC assay examples described herein meet the recommendations for best practices (5–7).

2. DOCUMENTATION

2.1. Standard Operating Procedure

Prior to establishing performance specifications (method validation), a fully optimized assay should be developed and documented in a written standard operating procedure (SOP). It is critical that this procedure is set and not changed prior to the validation process. This procedure should include all preanalytical, analytical and postanalytical processes associated with the method such as sample collection, sample handling, acceptable sample type, anticoagulants, sample preparation, instrument setup, sample acquisition, data analysis, and reporting. In addition, the SOP should describe the quality control procedures that should be used when performing the assay as well as limitations of the assays. Additional specific requirements for a method SOP

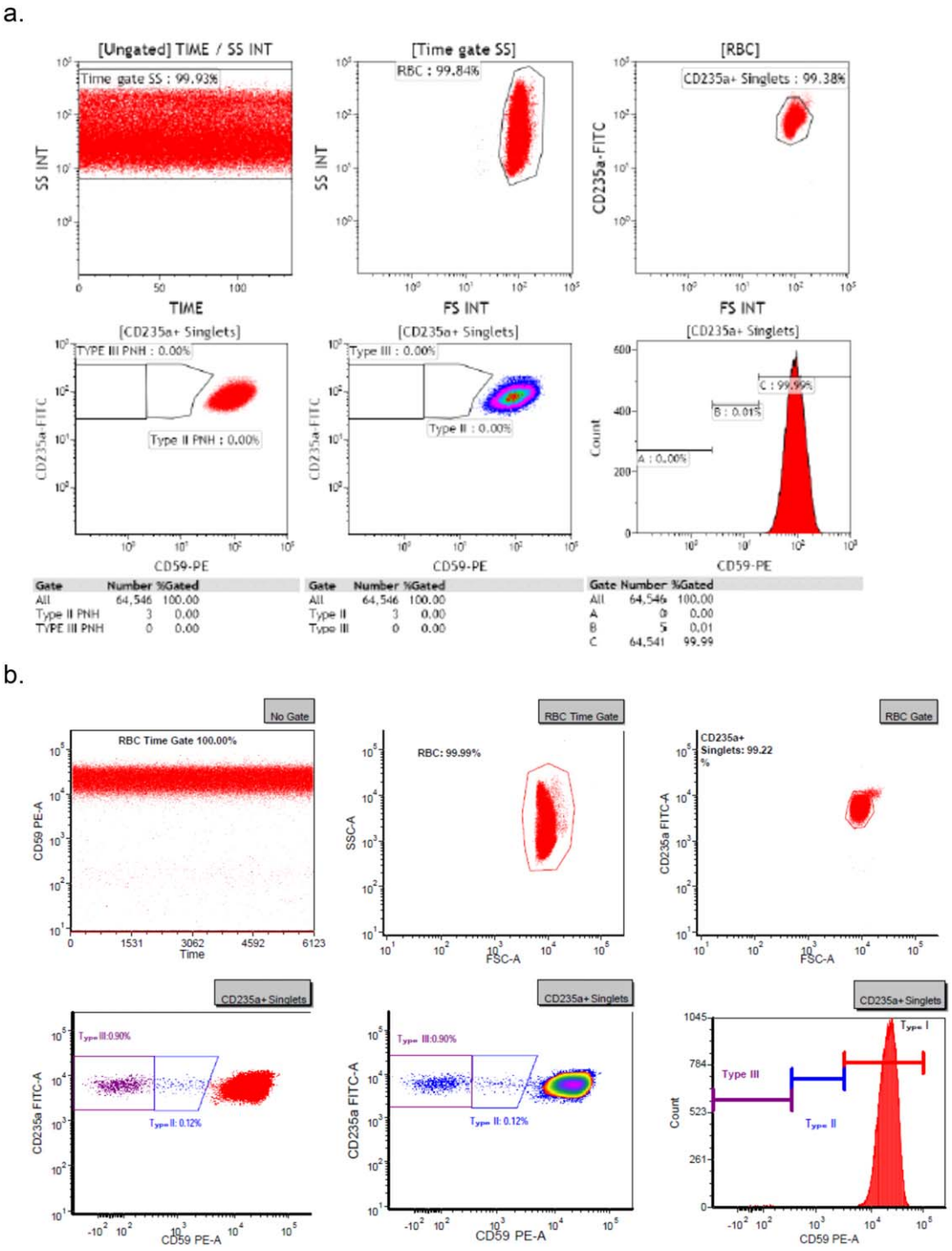

FIG. 1. Gating strategy for RBC PNH panel. Results from a healthy donor (**a**) and an abnormal (PNH positive) specimen (**b**) are shown. [Color figure can be viewed at wileyonlinelibrary.com]

depend on the regulatory environment in which the testing is conducted.

2.2. Validation Plan

The validation plan, also referred to as a validation protocol, provides detailed documentation of the validation experimental design. If a particular validation parameter is not applicable to the method, or cannot be addressed in the validation, an explanation should be provided in the validation plan. The analytical runs used to evaluate each of the required, and achievable, validation parameters must be described in the validation plan including the number of samples to be tested, the number of replicates, the number of analytical runs. Information

Table 2
WBC PNH Panel

Table 2a. Panel configuration

Fluorochrome	ALEXA488	PE	PE-Cy5.5	PE-Cy7	APC H7	V500
Experimental tube	FLAER	CD24 (clone ML5)	CD14 (clone MφP9)	CD15 (clone W6D3)	CD64 (clone 10.1)	CD45 (clone HI30)

Table 2b. Reportable Results

b. Quasi-quantitative reportable results

Population	Immunophenotype	Reportable result
PNH Neutrophils	$CD45^{dim}$, $CD15^{+}$, $CD24^{-}$, $FLAER^{-}$	Relative percentage (%) of neutrophils
PNH Monocytes	$CD45^{moderate}$, $CD64^{+}$, $CD14^{-}$, $FLAER^{-}$	Relative percentage (%) of monocytes

Qualitative reportable results

Population	Immunophenotype	Positive for 2 GPI markers	Negative for 2 GPI markers	Interpretation (PNH clone)
PNH neutrophils	SSC^{bright}, $CD45^{bright}$, $CD15^{+}$	Yes/No	Yes/No	Present/Absent
PNH monocytes	SSC^{dim}, $CD45^{bright}$, $CD64^{+}$	Yes/No	Yes/No	Present/Absent

Note that there is currently no single consensus panel for PNH Testing. The example shown is one of the recommended options[2], Validation data for this panel configuration is the assay is provided in Tables 3–9. [Color Table can be viewed at wileyonlinelibrary.com]

regarding the statistical data analysis and acceptance criteria should also be included. The validation plan will describe the source and type of validation samples, anticoagulants, sample age, the equipment (manufacture, model, serial number) and reagents (manufacturer, catalog number, and lot#). For monoclonal antibodies, the fluorochrome and clone designation must also be provided. The validation plan must also include the name and version of all software used in data sample acquisition and data analysis. The staff members conducting the validation should be listed in the validation plan.

Each laboratory must follow their institution's procedures for review, approval, and archiving of the validation plan.

2.3. Validation Report

The validation report describes the results of the experimental phase. The statistical tests described in the validation plan are applied to the data. Results are presented in the particular table and/or figure format, which best summarize the results. In addition, individual results should be presented in an appendix or available in a validation binder or electronic format. Copies of the gated data for every sample should also be available for review in a validation binder or electronic format.

It is not necessary that 100% of the data meet the acceptance criteria, but any results which do not, must be discussed in the validation report. Any deviations from the validation plan must be addressed in the validation report.

The lot number and expiration dates of all reagents use during the validation must be included in the report. If validation samples were purchased from a biologics vendor, the information regarding the samples should be provided in the validation report or be available in a validation binder or electronic file.

Each laboratory must follow their institution's procedures for review, approval, and archiving of the validation report.

Analytical method validation is not necessarily a one-time process. As the intended use of the data, or the regulatory requirements change, additional validation parameters may need to be evaluated in an extended or supplemental validation.

2.4. Assay Modifications

Modifications to the validated method such as changes in the antibody clones, fluorochromes, buffers, staining procedure, instrumentation, will also require additional validation. Comparability between the existing and modified method must be documented. The extent of the cross-validation depends upon the nature of the changes.

3. VALIDATION

Method validation is the confirmation by examination and the provision of objective evidence that the particular specifications for an intended use are fulfilled. To demonstrate that consistent results can be achieved, each testing laboratory must establish, or confirm, the performance specifications of the assay using their laboratory equipment, staff, and reagents (16,17).

The type of bioanalytical data generated in a method will dictate how the assay should be validated. Thus, the first step of an analytical method validation is determining which type bioanalytical data the method will generate: definitive quantitative, relative quantitative, quasi-quantitative, or qualitative (18) PNH assays typically report both qualitative and quasi-quantitative results. The presence or absence of a PNH clones (qualitative data) and size of the PNH clone (quasi-quantitative) in both RBC and WBCs are both reported. Understanding the concept of quasi-quantitative data is critical to designing the appropriate validation plan. Quasi means possessing certain attributes. In the case of quasi-quantitative bioanalytical data, continuous, numeric results are reported and there is a relationship between the test sample and the reported

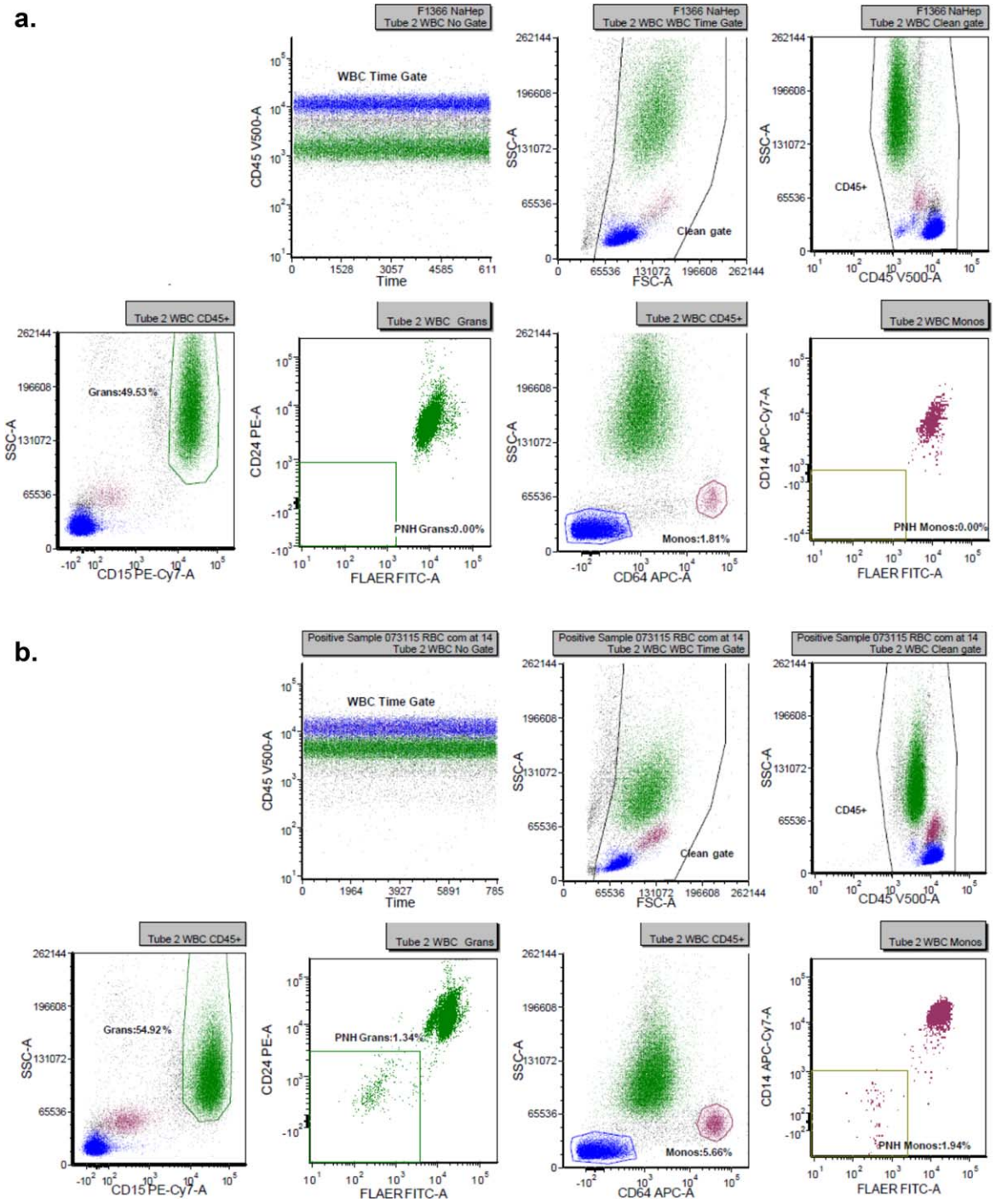

FIG. 2. Gating strategy for WBC PNH panel. Results from a healthy donor (**a**) and an abnormal (PNH positive specimen (**b**) are shown. [Color figure can be viewed at wileyonlinelibrary.com]

result but owing to the lack of calibrators and reference standards certain validation parameters notably accuracy and recovery cannot be demonstrated (11,12,18).

The next step of an analytical method validation is to clarify the intended use of the data and the associated regulatory requirements. By and large, PNH testing occurs in accredited clinical laboratories as the data are used for PNH diagnosis and treatment decision-making, thus the ICSH/ICCS guidelines for the validation of flow cytometric laboratory developed tests (LDT) should be followed (12). These guidelines recommend that the following validation parameters be evaluated: Accuracy/trueness, specificity, sensitivity, imprecision, linearity, carryover, measurement range/reportable range, stability,

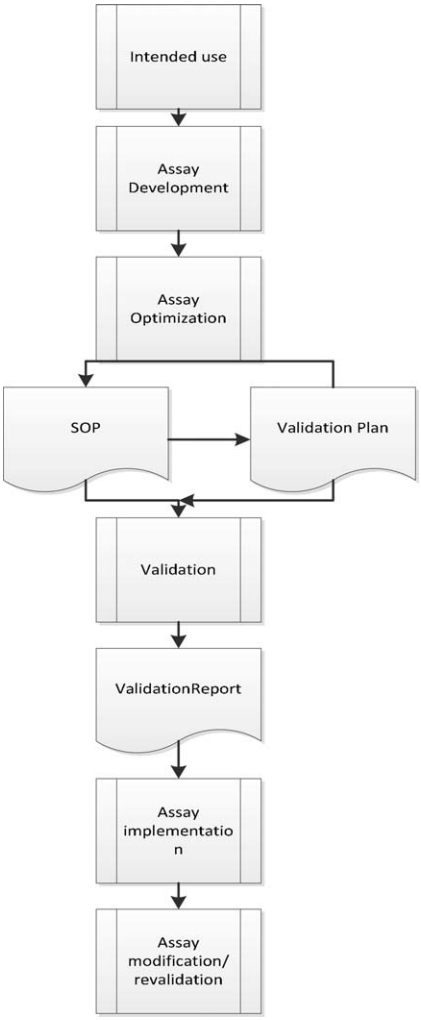

FIG. 3. Analytical method validation workflow. Overview of the recommended process for analytical method validation for flow cytometric methods. Prior to initiating the method validation, the intended use of the assay is established and assay development/optimization is completed. The method SOP is also completed prior to writing the validation plan and initiating the experimental phase. Finally, a validation report is prepared and the assay implemented. When necessary, revalidation occurs following the same process.

reference range. When PNH testing is included in a clinical trial, the data will likely also be used for patient decision-making and/or as an efficacy endpoint, thus a similar validation strategy should be followed.

While designing experiments to establish the required performance specifications for this assay, using valuable samples for more than one experiment is encouraged and can streamline the process. An example of this would be using the rare PNH samples (as well as nondiseased samples) for method comparison/accuracy as well as analytic sensitivity and specificity experiments, stability, and reproducibility/precision experiments.

3.1. Validation Samples

The recommended and most tested anticoagulant for PNH testing is EDTA; however, sodium heparin and ACD are also acceptable (5). Validation must be conducted for all anticoagulants, which the laboratory will accept for testing. If more than one anticoagulant will be used for the assay in clinical testing, each anticoagulant must be validated and be shown to be equivalent to the gold standard, which in this case is EDTA. This can be done by including samples from PNH patients and normal donor samples using other anticoagulants in the method comparison.

PNH method validations must include both PNH positive and negative samples. The negative samples can be obtained from the laboratory's patient population, an internal blood collection program, or from a commercial biologics vendor. A best practice is to obtain redirected PNH positive samples from an accredited clinical laboratory, which allows for comparison of results (19).

Because of the rarity of the disease and limited sample stability, PNH diseased samples for validation are difficult to obtain. Many laboratories will admix PNH positive samples with PNH negative in order to create validation samples with different levels of PNH. The same set of admixed samples can be used for sensitivity and precision assessments. These samples can also be used for linearity and inter-laboratory comparison, provided that dilutions are prepared accurately with reverse pipetting (6). The dilution series should include a minimum of five levels including an un-spiked sample. To achieve the desired percentage of PNH positive cells, measure the WBC of both the PNH positive and the nondisease state samples and the percentage of PNH positive cells in the disease-state sample. The targeted percentage of PNH positive cells are 0.001%, 0.005%, 0.01%, 0.1%, etc. The expected lower limit of quantification (0.01%) should fall in the middle of the dilution series. Verification of PNH assay sensitivity through a spiking experiment, currently in preparation as a module through the ICCS Quality and Standards Committee.

An alternative approach is to use PNH proficiency testing samples from such sources as the College of American Pathologist (CAP) or the United Kingdom National External Quality Assessment Service for Leucocyte Immunophenotyping (UK NEQAS LI) (20). However, it should be noted that these samples are fixed and may behave differently with respect to target antigen (epitope) expression and light scatter characteristics.

3.2. Accuracy/Trueness

According to the International Organization for Standardization (ISO), the accuracy of a method incorporates both trueness and precision. "Trueness" is defined as to the closeness of agreement between the average value obtained from a large series of measurements and the true value (if there is a standard) or accepted reference value of an analyte now referred to as "measurand" (the quantity intended to be measured) (20–22).

There are two reasons why accuracy/trueness by the ISO definition cannot be established for most flow cytometric methods. The first is that accuracy/trueness cannot be established for quasi-quantitative bioanalytical data (12). The second is that most flow cytometric

methods generate quasi-quantitative bioanalytical data owing to the fact that there are no qualitative reference standards. Nonetheless, the ICSH/ICCS Guidelines recommends some alternative approaches to satisfying the accuracy validation requirement for flow cytometric methods used in clinical laboratories (12). Each of these approaches are discussed below.

3.2.1. Assay of standard or reference materials

True cellular standard reference material is not available for any assay measuring cellular populations by Flow Cytometry (12). For PNH, UK NEQAS LI has developed proficiency testing material with a stability of several months which can be used to satisfy the Accuracy/Trueness validation parameter. Both PNH positive and negative samples must be evaluated.

3.2.2. Method comparison

For the diagnosis and monitoring of PNH, flow cytometric methods are considered the gold standard, thus comparison to another methodology is not an appropriate approach for addressing Accuracy/Trueness.

3.2.3. Interlaboratory comparison

Split sample testing with another laboratory is a feasible approach for demonstrating Accuracy/Trueness. To have a successful correlation, both laboratories should be using similar PNH assays, a comparison of the instrument setup procedures is advised. Both PNH positive and negative samples must be evaluated.

Per the typical alternative method comparison/interlaboratory comparisons recommendations 20 data points should be evaluated. The data points can be generated with 20 samples measured as single determinations or with fewer samples measured in triplicate. The advantage of the later approach is that it allows for a comparison of the precision achieved in each laboratory. Data from each laboratory can be compared for percent agreement; in addition, when replicates are tested the means ± SD can be compared.

In practice, comparison to a confirmed diagnosis will be the same approach to interlaboratory comparison for PNH accuracy validation. Both PNH positive and negative samples must be evaluated. NY State recommends that 15 normal and 3–5 PNH positive samples be evaluated, ideally covering a range of PNH clone sizes (19). It is necessary to follow state and federal requirements for validation sample size and all investigators undertaking a PNH validation are advised to confirm local and national regulations.

In this example (Table 3), when an exchange of three PNH positive specimens occurred between two laboratories, the mean percent difference between the two laboratories was 7% (range 4–9%) and 9% (range 5–17%), for PNH neutrophils and PNH monocytes, respectively. For the RBC Type II and RBC Type III percentages, the mean percent difference between the two laboratories was 20% (range 13–33%) and 9% (range 7–11%), respectively.

3.3. Specificity

Analytical specificity is defined as the ability of the assay to correctly identify or quantify an entity in the presence of interfering substances (22). Specificity for flow cytometric methods is addressed during the assay development or assay optimization phase. The selection of cellular markers, monoclonal antibody clones, reagent titration, wash steps, buffers, and gating strategy all influence the assays ability to measure only the cellular population-of-interest, i.e., specificity (3). Details around optimization of the PNH assay are addressed elsewhere (10). The validation process will establish and quantitate analytic specificity.

Table 3
Accuracy (Inter-Laboratory Correlation)

		Quasi-quantitative reportable results				Qualitative reportable results		
		%PNH				PHN interpretation (PNH clone)		
Population	Sample	Test method lab	Comparative method lab	% Difference	Mean % difference	Test method lab	Comparative method lab	Concordant
PNH neutrophils	S1	67.7	61.32	9%	7%	Present	Present	Yes
	S2	15.7	16.32	4%		Present	Present	Yes
	S3	5.51	4.9	9%		Present	Present	Yes
PNH monocytes	S1	85.41	89.73	5%	9%	Present	Present	Yes
	S2	23.41	24.73	6%		Present	Present	Yes
	S3	2.24	2.6	17%		Present	Present	Yes
RBC Type II	S1	2.05	2.31	13%	20%	Present	Present	Yes
	S2	0.07	0.08	14%		Present	Present	Yes
	S3	0.03	0.02	33%		Present	Present	Yes
RBC Type III	S1	30.63	27.91	9%	9%	Present	Present	Yes
	S2	10.63	9.87	7%		Present	Present	Yes
	S3	1.23	1.37	11%		Present	Present	Yes

Three samples were assayed as single determinations in each of two laboratories. Percent difference was calculated using the following formula:% Difference $= \left(\frac{\text{Test Method Lab} - \text{Comparative Method Lab}}{\text{Comparative Method Lab}}\right) \times 100$.

A number of publications have covered the topic of clinical specificity and sensitivity for PNH assays (5,6). The RBC PNH assay is generally considered less reliable than the WBC PNH assay for use in quantifying disease extent (clone size) due to the fact that in PNH patients, RBC may be hemolyzed and lost as a direct result of the disease process. The patient's PNH RBC lack CD59, in whole or in part, resulting in sensitivity to complement-mediated-hemolysis *in vivo*. In addition, blood transfusions, a common treatment for PNH, may dilute out the residual PNH RBC, so that the RBC clone is typically smaller than that typically detected in target WBC lineages

Analytical specificity for qualitative assays is more similar to clinical sensitivity than for quantitative methods in that it is defined as how well an assay performs with known negative and known positive samples. To establish the analytical specificity of the qualitative PNH assay, one must evaluate disease-state samples, ideally near the lower limit of detection (LOD) and the lower limit of quantification (LLOQ), as well as in nondisease-state samples. Data should be evaluated for concordance using the following formula where TN = true negative and FP = false positive:

$$Specificity = (TN \div (TN + FP)) \times 100$$

The CLSI Guideline for qualitative testing recommends 95% concordance however, for PNH testing an acceptance criterion of 100% is recommended due to the nature of the assay and the importance of the results (23). This acceptance criterion is achievable (Table 1, Supporting Information).

3.4. Sensitivity

According to the Clinical Laboratory Standards Institute (CLSI) guideline, sensitivity is defined as the "measured quantity value for which the probability of falsely claiming the absence of a measurand is β, given a probability α of falsely claiming its presence." (24) More simply stated, sensitivity is defined as the ability of the assay to distinguish signal from background and precisely measure low amounts of the measurand. For assays designed to measure rare event analysis defined as occurring at frequencies of $\leq$1% such as PNH or cellular depletions, it is necessary to establish the limit of blank (LOB)/limit of detection (LOD) and the lower limit of quantification (LLOQ)**.** (11,25)

3.4.1. Sensitivity - analytical (LOB/LOD)

The LOB is defined as the highest apparent signal expected in the absence of the measurand (i.e., a blank sample), whereas; the limit of detection (LOD) is defined as the ability to detect the measurand at a level that can reliably be distinguished from the LOB where 95% of low levels samples will be detected above the LOB. LOB is calculated as the Mean of the blank + 1.645 SD. The LOD can be calculated as the Mean of the blank + 3 SD. Ideally 10 samples will be used to for this calculation (12,25,26).

The first challenge in establishing the LOB is establishing the optimal "blank" samples, similar to the "buffer blank" used in spectrophotometric methods. Two approaches to creating the blank have been reported (11,12). One approach is to create "blank" samples by partially staining a sample by omitting antibodies in such a way that the population-of-interest will not be detected. This can be achieved by omitting the last one or two antibodies in the gating hierarchy. The second is to fully stain samples obtained from patient populations known to be negative for the cellular population, or cellular antigen, of interest. In the case of PNH the population of interest is not typically present in samples from healthy individuals, thus samples from apparently healthy donors can be used to establish the LOB/LOD.

Analytical sensitivity (LOD) was calculated from data generated using 10 PNH negative samples by calculating the mean valued plus 3SD (Supplemental information Figure 1a–c. Note that PNH negative specimens are positive for CD235a++ RBC, and Type-I RBC parameters, thus the LOD was not calculated for these parameters. When approximately 1,000,000 RBC were counted using the gating strategy in Figure 1, the mean number of Type III RBC events from 10 healthy donors was 2 with a standard deviation of 1 (Table 2, Supporting Information). Thus, the calculated LOB/LOD for the RBC assay was 4 and 6 events, respectively, which corresponded to 0.0006% and 0.0009%. The same approach was used to establish the LOB/LOD for the WBC assay was used. For PNH neutrophils the LOB/LOD was 8 and 11 events, respectively, which corresponded to 0.0015% and 0.0023% (Table 2, Supporting Information). For PNH monocytes the LOB/LOD was 3 and 4 events, respectively, which corresponded to 0.005% and 0.007% (Table 2, Supporting Information). The total number of acquired events for neutrophils and monocytes in this data set was ~500,000 and 50,000, respectively.

3.4.2. Sensitivity—functional (LLOQ)

The lower limit of quantification (LLOQ) is defined as the lowest concentration that can be reliably detected with acceptable accuracy and precision (18). For flow cytometric methods, the LLOQ can be established by analyzing samples with levels of the measurand approaching the LOB/LOD. A minimum of 5 levels should be tested in replicates of 3–5.

The acceptance criteria for assay imprecision are determined by the intended use of the data. Although assay imprecision in the range of 20–35% coefficient of variation (CV) has been reported to be acceptable at the LLOQ of flow cytometric biomarker methods, each laboratory must determine the tolerability limits appropriate for each assay and define it in the Method Validation Plan (11,22).

When establishing the LLOQ it is important to evaluate the number of events in the gate in addition to the

reportable result. The LLOQ will be the lowest value where the results for each replicate are above the LOD, there is evidence of a titration effect, and acceptable precision is achieved. In an interlaboratory comparison of the ERIC CLL MRD assay, good correlation was observed when CLL 20–50 events were present (20). That said, there is no requirement for a minimum number of clustered events (21,27,28).

3.5. Imprecision

Assay imprecision or repeatability is defined as the closeness of agreement between independent test results obtained under stipulated conditions (22) and is usually expressed at the standard deviation (SD) or the coefficient of variation (CV%). Intraassay imprecision (within run), interassay imprecision (between run) should be established. In addition, interoperator and interinstrument variation should be evaluated.

3.5.1. Intraassay imprecision

Three to five samples with varying levels of PNH and including at least one PNH negative samples as described in Section 3.1 should be used to assess intra-assay imprecision (11,19). Each sample should be assayed in triplicate in a single analytical run, meaning three staining tubes per sample and three acquisition files. The mean, SD and %CV for each sample and each reportable result should be calculated. The overall intra-assay imprecision should be reported and the mean %CV (and range) achieved for all samples, excluding the PNH negative samples. The acceptance criterion for imprecision for flow cytometric methods varies and is dependent on the abundance of the reported cellular population (11,12).

An example of precision data is provided in Table 4. For all reportable results, Type-II RBC relative percentage (%), Type-III RBC relative percentage (%), PNH Neutrophils relative percentage and PNH Monocytes relative percentage (%), the intra-assay precision was $\leq$10% CV.

3.5.2. Inter-assay imprecision

When assessing inter-assay imprecision the experimental design must avoid the contribution of specimen stability. For this reason, inter-assay imprecision is often conducted using a commercial available flow cytometry preserved whole blood quality control (QC) material or proficiency testing (PT) samples. Owing to the limited stability of PNH samples, and the lack of PNH cells in flow cytometry QC, inter-assay imprecision for PNH assays should be done on PT samples as described in Section 3.1. Another approach is for the one analyst to prepare two sets of replicates for each sample and then acquire the samples on separate instruments on the same day.

Two to three samples should be assayed in triplicate in a minimum of four analytical runs. For each sample and each reportable result, the mean of the replicates will be calculated for each analytical run. The mean of daily means, SD (of daily means), and %CV (of all runs), will also be calculated. The overall inter-assay imprecision should be reported and the mean inter-assay %CV (and range) achieved for all samples, excluding PNH negative samples. Although acceptance criteria for imprecision for flow cytometric methods range from 10–30%CV, inter-assay precision of $<$10%CV for high-sensitivity PNH assays have been achieved. If assay imprecision of samples in this range greatly exceeds 10%CV, the laboratory should conduct an investigation. Note that for samples approaching the assay LLOQ, greater variability is acceptable (11,18).

3.5.3. Inter-operator variation

Inter-analyst variation can be addressed in a variety of ways, in all scenarios each operator must be processing the same samples, if the samples are whole blood samples, the samples must be processed on the same day.

Data analysis can include calculating the %CV of all replicates. The %CV achieved by each operator can also be compared. The mean value generated by each operator can also be compared.

3.5.4. Inter-instrument variation

Inter-instrument variability can be evaluated by combining the between-instrument and between-run variability of the results achieved during the inter-assay precision experiments. Results must indicate that comparable assay performance between instruments has been achieved.

Data analysis can include comparing both the imprecision and the mean values obtained from each instrument.

Table 4
Intra-Assay Imprecision

	Quasi-quantitative reportable results				Qualitative reportable result			
	%PNH				PNH Interpretation (PNH clone)			
Population	Replicate 1	Replicate 2	Replicate 3	CV (%)	Replicate 1	Replicate 2	Replicate 3	Concordant
PNH neutrophils	1.72	1.95	1.66	7	Present	Present	Present	Yes
PNH monocytes	2.33	2.79	2.57	7	Present	Present	Present	Yes
RBC Type II	0.28	0.26	0.22	10	Present	Present	Present	Yes
RBC Type III	3.42	3.77	3.99	6	Present	Present	Present	Yes

A sample was assayed in triplicate in a single analytical run.

3.6. Linearity

Linearity verification is not applicable for quasi-quantitative methods (26). (Only when the fluorescence intensity signal output is quantified using fluorescence calibration/quantitation beads are flow cytometric results considered as relative quantitative in which case linearity of the quantitation beads will be evaluated (29).)

Although in general, linearity verification is not applicable for quasi-quantitative methods, (26) the results obtained with the dilution series samples can be evaluated for linearity but in this case no acceptance criteria should be applied as the data are dependent on pipetting accuracy when preparing the dilutions and no calibrated reference material is available plotted.

Note that instrument linearity can be demonstrated and should be verified biannually as part of the instrument quality control (QC) (14).

3.7. Carryover

For high-sensitivity assays reporting rare events, such PNH assays, it is critical to evaluate carryover. This can be accomplished in a variety of ways. One way is to acquire a tube of buffer after each sample and assess the carryover. Note that carryover should also be addressed during the sample testing phase as described below.

3.8. Measurement Range/Reportable Range

Reportable range is defined as the acceptable limits that each of the reportable analytes has met the analytical precision requirements. This is addressed with the by establishing the analytical sensitivity (LOB/LOD) and functional sensitivity (LLOQ).

3.9. Reference Interval

The value of establishing a reference interval for PNH testing is questionable as it is well established that RBC and WBC displaying a PNH phenotype are not found in healthy individuals or in other disease states. Data from the non-PNH samples used in the method validation can be compiled to estimate a reference interval (30). Note that some accreditation agencies require that 25 samples obtained from apparently healthy volunteers are evaluated yearly (19,31). Data from these samples can also be used to establish and re-assess the analytical sensitivity (LOB/LOD).

Background of PNH events in normal samples have been reported to contain up to 0.0006% Type III PNH RBC phenotypes and 0.01% PNH neutrophils phenotypes using a high-sensitivity PNH assay (6). If values in normal samples greatly exceed this target, the laboratory should conduct an investigation.

3.10. Stability

Stability is defined as the capability of a sample material to retain the initial property of a measured constituent for a period of time within specific limits when the sample is stored under defined conditions (32).

3.10.1. Specimen stability assessment

Current recommendations for stability assessment for flow cytometric methods are that a minimum of five samples should be tested within 2 h of collection and at relevant time-points thereafter (12,33). A best practice is to evaluate one time-point past the expected stability window.

Stability assessment is the most challenging aspect of method validation. With flow cytometric methods, stability must be demonstrated for both the cellular population of interest as well as the cellular antigens. PNH assays present additional challenges with regard to specimen stability. Like their normal counterparts, the GPI-deficient neutrophils are fragile and can deteriorate rapidly after sample collection and are extremely sensitive to sample handling/storage conditions, changes in antigen expression in normal cells must be thoroughly evaluated so that PNH cells are not confused with unstable antigen expression on normal cells.

PNH testing often occurs in a reference laboratory setting where specimens are evaluated 24 h or more postcollection, in this scenario relevant time-points for stability assessment would be at baseline (within 2), 24, 48, and 72 h postcollection. Given that PNH stability has been previously reported at 48 h postcollection if stored at 4°C, the evaluation of additional time-points between 24 and 48 h is also recommended. Note that, if fresh samples are not available, a later time-point is acceptable for the baseline sample. A minimum of five PNH positive samples and two PNH negative samples should be evaluated. Ideally, stability assessment will include PNH samples near the LLOD/LLOQ.

Statistical analysis should include calculating the percent change from baseline as well as the coefficient of variation between the baseline sample and each time-point. The acceptance criteria are a percent change of no $>20\%$ and/or a variation of $\leq 25\%$CV in 80% of the samples (12,33). In addition to the statistical analysis, it is mandatory to assess changes in light scatter and antigen expression levels.

Given that PNH phenotypes are defined by a lack of expression of CD59 on RBC or lack FLAER binding and CD24 or CD157 on neutrophils or FLAER and CD14 or CD157 on monocytes the stability of these markers in normal cells must also be established. Thus, the stability evaluation should include two to three samples from apparently healthy donors.

3.10.2. Processed specimen stability

The stability of the processed (stained/fixed) specimens prior to acquisition should also be evaluated. In this case, the time-points evaluated are dependent on the practices and workload of each laboratory but must encompass the maximum anticipated delay between processing and acquisition. The statistical analysis, data evaluation, and acceptance criteria for processed specimen stability must be the same as those applied to whole blood specimen stability

(12,33). If processed specimen stability is not documented, specimens should be acquired within 1 h.

3.10.3. Cocktail stability

The use of premixed antibody cocktails in the PNH assay is mandatory to obviate the risk of pipetting errors that can result in the generation of PNH phenotypes. However, it is critical to demonstrate that equivalent results are achieved when using a stored cocktail as when individually pipetting reagents into a tube. This comparison will identify loss of fluorescence intensity or diminished staining due to tandem-conjugate breakdown, photo bleaching, quenching, fluorophore interactions, or other changes resulting from the mixture condition.

Cocktail stability validation is accomplished by comparing the original cocktail to a freshly prepared cocktail using paired samples. Alternatively, the original cocktail can be compared to the individually pipetted single antibodies using paired samples. A best practice is to include both PNH positive samples and samples from apparently healthy donors. Cocktails should to be evaluated every week or bi-weekly for the duration of the stability study.

The statistical analysis, data evaluation, and acceptance criteria for processed specimen stability should be the same as those applied to whole blood specimen stability (12,33). In addition, the fluorescence intensity of each component antibody in the cocktail must be compared to the fluorescence of the corresponding fresh cocktail or manually pipetted antibody.

4. INTERNAL QUALITY CONTROL (IQC)

Regulatory agencies require the following control procedures: instrument internal quality control (IQC) which include optical alignment, protocol standardization, inter-instrument correlation, compensation; assay process controls; positive and negative internal controls; critical reagent new lot phase-in controls. Documentation of acceptability criteria for all control procedures and corrective action is required (25,34–37).

4.1. Instrument IQC

A well maintained, optimized and standardized flow cytometer will ensure that for each specimen identical fluorescent and light scatter output signals are obtained. In addition, a robust IQC system provides an "early warning system" to potential problems. Any anomalies should be documented and investigated fully before proceeding with testing patient material.

A common IQC material for flow cytometers is the use of microbeads to monitor compensation, fluidics, laser, and PMT voltages. Results should be plotted on a Levy–Jennings type plot and any noticeable drift should be investigated immediately. To do this, boundaries of acceptability should be established and all staff be made aware and to understand what these boundaries mean. It is also important that the instrument is checked with beads and stabilized samples following instrument servicing to re-establish optimal settings. Furthermore, the laboratory undertaking the test should fully understand the technical protocols designed for PNH flow cytometric testing.

4.1.1. Optical alignment

Flow cytometry instrument QC consists of verifying the optical alignment of the instrument. Proper alignment of the sample stream with the laser(s) and respective optical detectors is critical to obtain accurate and reproducible results. This is performed by using alignment microspheres with a uniform signal for each light scatter and fluorescent parameter. The goal of alignment is to obtain the "brightest and tightest" signal with maximal signal and minimal variation. Peak or median channels for forward scatter (FSC) and side scatter (SSC) and all relevant fluorescent channels used in the respective protocol are captured along with their respective CV values.

The manufacturer will optimize the laser alignment and establish optimum target values for light scatter and fluorescence during the initial instrument installation. The testing laboratory will then perform a daily instrument QC and verify that the beads in each channel fall within the pre-established target range (13–15,38–40).

4.1.2. Standardization

The goal of standardizing the flow cytometer daily is to ensure that any observed changes in the populations being analyzed are not due to instrument variation. Standardization is performed by running stabilized microspheres with known fluorescence and light scatter characteristics. Each parameter is placed into pre-established channels (target channels established at instrument setup) by adjusting the detector voltage settings. Voltage settings for each parameter are then captured and monitored for fluctuations. Longitudinal displays of changes in voltage can be graphically displayed in Levy-Jennings type plots to visualize shifts and/or trends in the instrument performance (13,14,39,40).

4.1.3. Compensation

Compensation, the subtraction of spectral overlap, is directly related to instrument variables such as fluorescence PMT voltages, gains, laser power, and optical filters. A change in these variables can impact compensation setting. Please note that compensation for FITC is not a substitute for compensating with FLAER Alexa 488. Compensation verification must be part of the daily assay QC (13,39).

4.1.4. Instrument carryover

Testing should be performed to measure the level of carryover from one sample to the next and provide evidence that carryover does not exceed specifications. This is particularly important for laboratories performing PNH and other rare event analysis, specifically for those instruments using a carousel or multi-well sampler. The preferred method for assessing carry over is to acquire a

sample with high levels of PNH positive clones followed by a sample from an apparently healthy volunteer (12,41). If carry over is evident, a best practice is to acquire a buffer tube between clinical samples.

4.2. Assay IQC

When testing clinical samples, assay specific QC checks are required for the monitoring of instrument setup (voltage and compensation), reagent integrity, and successful staining. Target values and achieved results for the QC checks must be fully documented (37).

A challenge in PNH testing is finding appropriate process control material. Not only are there no PNH-specific commercially available quality controls but the use of patient samples as a positive control is not feasible as PNH is a rare disease so most laboratories do not see samples regularly. Alternative strategies for assay monitoring include the use of internal controls or non-PNH samples (commercially available flow cytometry QC material or whole blood from healthy donors).

Internal controls, i.e., normal leucocytes and normal red blood cells are always present in each specimen tested, are useful in verifying that the staining reagents were appropriately added, staining processes were performed properly and that the instrument setup was optimal for PNH assays.

For WBC assays, normal neutrophils will typically show bright expression of the neutrophil/granulocyte gating marker CD15, while 'internal control' lymphocytes will not stain. Normal neutrophils will exhibit bright staining with FLAER and antibodies to GPI-linked structures such as CD24 and CD157. Normal lymphocytes (internal positive control) stain brightly with FLAER. B-lymphocytes (internal positive control) stain with CD24, while T-lymphocytes do not and thus serve as an 'internal negative control' for this reagent. For samples containing PNH lymphocytes, such are not stained with either FLAER or CD24. For PNH samples assessed with CD157-based assays, lymphocytes serve as an internal negative control.

Normal monocytes will typically stain brightly with CD64 conjugates, while once again, lymphocytes serve as an internal negative control for this monocyte gating reagent. Normal monocytes stain brightly with FLAER and with antibodies to GPI-linked structures such as CD14 and CD157. As above, normal lymphocytes serve as internal positive control for FLAER and internal negative controls for CD14/CD157. Examples of a normal and a PNH sample stained with a cocktail comprising FLAER, CD24, CD15, CD64, CD14, and CD45 are shown in Figures 2a,b, respectively. From these examples it is apparent that although lymphocytes are not useful target cells for diagnosing and measuring the extent of PNH,

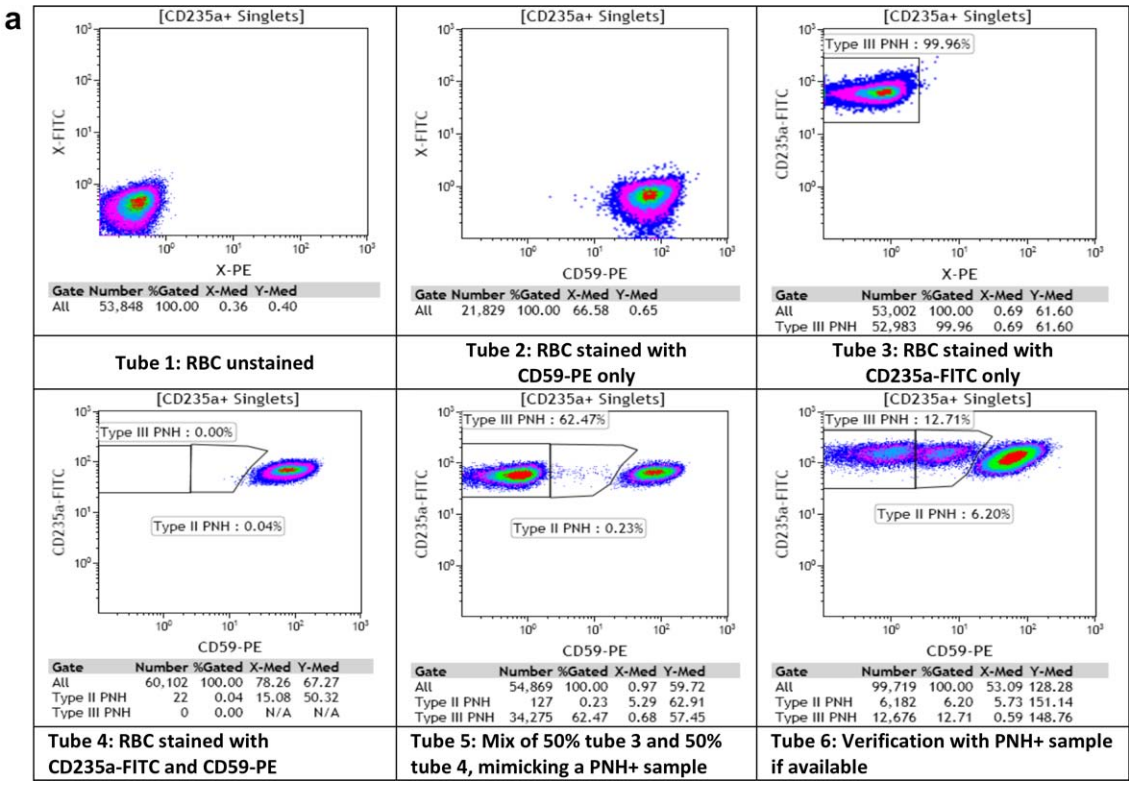
a
[CD235a+ Singlets]
X-FITC
X-PE
Gate Number %Gated X-Med Y-Med
All 53,848 100.00 0.36 0.40
Tube 1: RBC unstained
[CD235a+ Singlets]
X-FITC
CD59-PE
Gate Number %Gated X-Med Y-Med
All 21,829 100.00 66.58 0.65
Tube 2: RBC stained with CD59-PE only
[CD235a+ Singlets]
Type III PNH : 99.96%
CD235a-FITC
X-PE
Gate Number %Gated X-Med Y-Med
All 53,002 100.00 0.69 61.60
Type III PNH 52,983 99.96 0.69 61.60
Tube 3: RBC stained with CD235a-FITC only
[CD235a+ Singlets]
Type III PNH : 0.00%
Type II PNH : 0.04%
CD235a-FITC
CD59-PE
Gate Number %Gated X-Med Y-Med
All 60,102 100.00 78.26 67.27
Type II PNH 22 0.04 15.08 50.32
Type III PNH 0 0.00 N/A N/A
Tube 4: RBC stained with CD235a-FITC and CD59-PE
[CD235a+ Singlets]
Type III PNH : 62.47%
Type II PNH : 0.23%
CD235a-FITC
CD59-PE
Gate Number %Gated X-Med Y-Med
All 54,869 100.00 0.97 59.72
Type II PNH 127 0.23 5.29 62.91
Type III PNH 34,275 62.47 0.68 57.45
Tube 5: Mix of 50% tube 3 and 50% tube 4, mimicking a PNH+ sample
[CD235a+ Singlets]
Type III PNH : 12.71%
Type II PNH : 6.20%
CD235a-FITC
CD59-PE
Gate Number %Gated X-Med Y-Med
All 99,719 100.00 53.09 128.28
Type II PNH 6,182 6.20 5.73 151.14
Type III PNH 12,676 12.71 0.59 148.76
Tube 6: Verification with PNH+ sample if available

FIG. 4. (**a**) Suggested PNH RBC assay monthly control for verification of voltage setting, compensation and location of GPI-negative population. **(b)** Suggested WBC RBC assay monthly control for verification of voltage setting, compensation, and location of GPI-negative population.

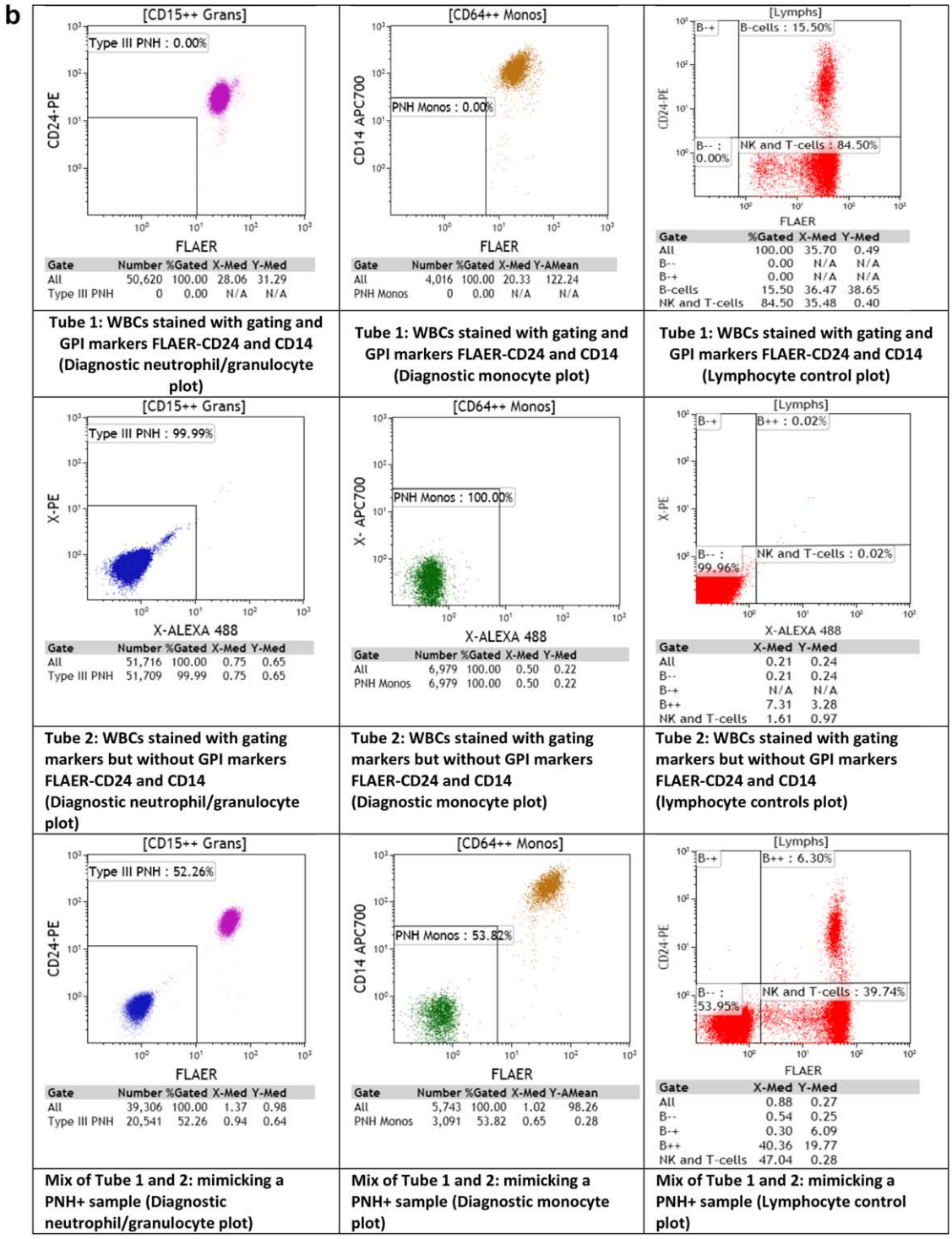
b
[CD15++ Grans]
Type III PNH : 0.00%
CD24-PE
FLAER
Gate Number %Gated X-Med Y-Med
All 50,620 100.00 28.06 31.29
Type III PNH 0 0.00 N/A N/A
[CD64++ Monos]
PNH Monos : 0.00%
CD14 APC700
FLAER
Gate Number %Gated X-Med Y-AMean
All 4,016 100.00 20.33 122.24
PNH Monos 0 0.00 N/A N/A
[Lymphs]
B-+
B-cells : 15.50%
CD24 PE
B-- : 0.00%
NK and T-cells : 84.50%
FLAER
Gate %Gated X-Med Y-Med
All 100.00 35.70 0.49
B-- 0.00 N/A N/A
B-+ 0.00 N/A N/A
B-cells 15.50 36.47 38.65
NK and T-cells 84.50 35.48 0.40
Tube 1: WBCs stained with gating and GPI markers FLAER-CD24 and CD14 (Diagnostic neutrophil/granulocyte plot)
Tube 1: WBCs stained with gating and GPI markers FLAER-CD24 and CD14 (Diagnostic monocyte plot)
Tube 1: WBCs stained with gating and GPI markers FLAER-CD24 and CD14 (Lymphocyte control plot)
[CD15++ Grans]
Type III PNH : 99.99%
X-PE
X-ALEXA 488
Gate Number %Gated X-Med Y-Med
All 51,716 100.00 0.75 0.65
Type III PNH 51,709 99.99 0.75 0.65
[CD64++ Monos]
PNH Monos : 100.00%
X- APC700
X-ALEXA 488
Gate Number %Gated X-Med Y-Med
All 6,979 100.00 0.50 0.22
PNH Monos 6,979 100.00 0.50 0.22
[Lymphs]
B-+
B++ : 0.02%
X PE
B-- : 99.96%
NK and T-cells : 0.02%
X-ALEXA 488
Gate X-Med Y-Med
All 0.21 0.24
B-- 0.21 0.24
B-+ N/A N/A
B++ 7.31 3.28
NK and T-cells 1.61 0.97
Tube 2: WBCs stained with gating markers but without GPI markers FLAER-CD24 and CD14 (Diagnostic neutrophil/granulocyte plot)
Tube 2: WBCs stained with gating markers but without GPI markers FLAER-CD24 and CD14 (Diagnostic monocyte plot)
Tube 2: WBCs stained with gating markers but without GPI markers FLAER-CD24 and CD14 (lymphocyte controls plot)
[CD15++ Grans]
Type III PNH : 52.26%
CD24-PE
FLAER
Gate Number %Gated X-Med Y-Med
All 39,306 100.00 1.37 0.98
Type III PNH 20,541 52.26 0.94 0.64
[CD64++ Monos]
PNH Monos : 53.82%
CD14 APC700
FLAER
Gate Number %Gated X-Med Y-AMean
All 5,743 100.00 1.02 98.26
PNH Monos 3,091 53.82 0.65 0.28
[Lymphs]
B-+
B++ : 6.30%
CD24 PE
B-- : 53.95%
NK and T-cells : 39.74%
FLAER
Gate X-Med Y-Med
All 0.88 0.27
B-- 0.54 0.25
B-+ 0.30 6.09
B++ 40.36 19.77
NK and T-cells 47.04 0.28
Mix of Tube 1 and 2: mimicking a PNH+ sample (Diagnostic neutrophil/granulocyte plot)
Mix of Tube 1 and 2: mimicking a PNH+ sample (Diagnostic monocyte plot)
Mix of Tube 1 and 2: mimicking a PNH+ sample (Lymphocyte control plot)

FIG. 4. Continued

they serve a vital function in monitoring antibody performance and appropriate instrument setup for each sample analyzed (6,9).

General flow cytometry QC material can also be used as full process control provided that the laboratory established target values and acceptance criteria for each antigen used in the assay. Ideally, non-PNH samples are best for process control.

Alternatively, whole blood from healthy donors can be stained in two different manners.

- RBC QC: The first tube is stained with both CD235a (gating marker) and CD59 (GPI marker). The second tube is stained only with CD235a (gating marker) thus mimicking a PNH sample. In an additional step, both tubes can be mixed to verify that the assay will separate PNH cells (CD59-negative) and normal cells (CD59-positive cells in an approximate 50%/50% distribution. (Fig. 4a).
- WBC QC: The first tube is stained with all markers (gating and GPI markers in the assay (CD45, CD15, CD64, FLAER, and CD14 and CD24 or CD157) for the evaluation of the normal expression of all markers. Whereas the second tube is stained with only the gating markers (CD45, CD15, and CD64) but not the GPI markers in use (FLAER, CD24 and CD14 or FLAER and CD157) thus mimicking a PNH sample. As an additional step, both staining tubes can be mixed and used to verify that the assay will distinguish PNH and normal populations or even to verify the LLOQ. (Fig. 4b).

It is also important to have assay specific QC checks when testing clinical samples. This may involve using a Delta Check to compare results from prior samples tested for PNH clones. Acceptably limits for the process control and delta check should be defined (37).

Each new lot of antibody, critical non-antibody reagent (such as homemade buffers or lysing agents) must be evaluated for equivalency prior to putting in use for testing patient sample (36).

5. EXTERNAL QUALITY ASSESSMENT/PROFICIENCY TESTING

The role and benefits of external quality assessment/proficiency testing in laboratory medicine with regards to improving the quality of clinical results and monitoring fitness for clinical purpose are well known and have been described previously (42,43). Because of the rare incidence of PNH all laboratories performing PNH testing should be members of an EQA/PT program.

It is important to note that over time any laboratory can experience a result that is unsatisfactory/out of consensus. Any such incident should be thoroughly investigated locally (with the assistance of the EQA/PT provider if necessary) to identify the root cause of the aberrant result and to identify preventative and corrective actions to ensure that the incident does not recur. All investigations into aberrant results should be recorded and retained and any patients tested over the period of unsatisfactory performance should be reviewed to ensure the validity of results.

Where EQA/PT results are within consensus/acceptable limits the data should be reviewed upon receipt to check for potential trends and as a tool to identify any methodological variations that could adversely affect/benefit the testing process.

5.1. Ensuring and Assessing Staff Competency

Establishment of ongoing competency assessments is an essential part of good laboratory practice and is an essential requirement for laboratories seeking accreditation to ISO 15189 (44,45) As such all staff performing flow cytometric testing should be properly trained and competent. When assessing the competency of staff with regards to PNH testing by flow cytometry the following areas should be considered:

- Knowledge of the condition.
- Knowledge of the characteristics of the target cells.
- Knowledge of the testing process i.e. antibody selection, staining technique, gating methodologies.

These guidelines contain all of the relevant information to meet a sufficient level of understanding in the areas outlined above. However, it should be noted that whilst guidelines can be used in standard operating procedure production and training of staff they do not impart competency. The laboratory is responsible for ensuring that staff competency monitoring occurs at regular intervals against locally predefined evaluation criteria.

Potential methods for assessing staff competency have been outlined elsewhere and should be adapted to provide a consistent inter-laboratory approach to staff competency assessment (44). However, the rare incidence of PNH means it is likely that many laboratories will not see cases on a regular basis and as such ensuring staff competencies with regards to PNH flow cytometric data analysis and staining pattern recognition can become difficult. As such, it is recommended that laboratories:

- Perform intra-laboratory staff analysis comparisons using FCS/LMD files from PNH+ cases.
- Create networks to allow access to anonymized FCS/LMD files of PNH cases for use in competency assessment.

If access to suitable PNH samples or FCS files is not available, then external quality assessment/proficiency testing materials can be used as part of competency procedures. Alternatively, pseudo-PNH samples can be manufactured within laboratories by omitting the addition of the GPI-linked antibodies when staining a normal sample (6) (A sample stained in this way could then be used to create different levels of PNH for competency purposes—although due to the nature of production it is important to note that for red blood cell analysis this method would produce only Type III red blood cells, and as such is not an exact substitute for genuine material/FCS data files).

6. SUMMARY

The validation of flow cytometric assays for PNH clone detection is particularly challenging. Major challenges arise from the fact that clinical samples for validation of this rare disease are difficult to obtain. This is a particular challenge when validating samples stability. Once the assay is validated and performance specifications are established, internal quality control and external quality assurance ensures that these performance specifications can be consistently met over time.

Developing and sustaining staff competency is critical to successfully performing the high sensitivity assay.

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